VARIABILITY OF DRY MASS AS A FUNDAMENTAL BIOLOGICAL PROPERTY DEMONSTRATED FOR THE CASE OF VACCINIA VIRIONS

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ABSTRACT The dry mass of individual vaccinia virions, as an example of a presumably uniform biological population, prepared in different lots at the Institute for Tropical Hygiene in Hamburg, was tested for variability by quantitative electron microscopy. A value of 5.26×10^{-15} g for the median weight of the particle was calculated from 7,300 determinations. By assessing the variability of polystyrene latex spheres, which were used as mass standards, we demonstrated that the variability of dry mass of vaccinia virions is fivefold greater than the variability (standard) introduced by the method for determining mass. It was concluded that while genetic control in a presumably homozygous virion is strict with respect to quality, quantity of viral components (other than DNA) varies in fashion that can be aptly described by a log-normal distribution.

It is recognized that this observation is empirically supported by the paradigm that any composite biological entity is subject to quantitative variability, the more so the heavier the individual representatives of a species are.

In addition, the effects that extractions and staining have on the dry mass of vaccinia are reported, as well as is the median for the dry mass of other strains of pox viruses.

INTRODUCTION

Shortly after development of the theoretical and instrumental basis for mass determinations from measurements of contrast in the electron microscope (4–6, 13–15), a series of biological objects was studied with the intent of testing the applicability of the technique to small (10^{-18} g) and relatively large (10^{-12} g) structures. Among these objects were bull spermatozoa, for which log-normal distributions of the dry mass of the head and other structural components were found.[1] The variability of the dry mass of the heads was of particular interest, because constancy of DNA had to be assumed in accordance with existing biological information and thinking. Thus, only the non-DNA components of the sperm head could vary, and since the specific density of a population of heads in an ejaculate is rather constant (8), it was concluded that enclosing membranes and other structures of the head varied along with

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The opinions or assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

[1]Bahr, G. F., H. Gütter, W. F. Engler, and B. Schell. 1978. The combined use of high vacuum levels and cooling as anticontamination measures. Manuscript submitted for publication.

the contents of the head. Furthermore, variability of the sperm's tail structures was found to be uncoordinated with the variability of the sperm head; i.e., a heavy head could have a light tail, and a small and light head could be found attached to a very heavy tail structure. The situation suggested that while genetic control in macro-molecular assembly must be assumed strict with respect to quality, it is lax in regard to quantity. The present study was undertaken with the aim of analyzing macromolecular assembly of a biological structure several orders of magnitude in mass below bull spermatozoa but heavier and more complex than assemblies capable of crystallization.

Early phases of this research came to a halt when the influence of specimen contamination caused by the electron microscope could not clearly be eliminated from the observed variability of mass among viruses. Contamination can now be reduced to negligible levels, however, in electron microscopes with high vacuum at the specimen level[2] and with other anticontamination devices. Recent acquisition of such a commercially available instrument (Zeiss EM 10 [4]) allowed us to repeat mass measurements on vaccinia virions and thus to reopen the issue.

MATERIALS AND METHODS

Virus Preparation for Electron Microscopy

Vaccinia virus was cultivated according to Hoagland et al. (6) in rabbit skin as has been previously described (11). Virions were prepared from homogenates of infected tissue in McIlvaine buffer solution diluted 1:50, pH 7.3, to which 5% (vol/vol) trifluortrichlorethylene (Freon 113) had been added. Differential centrifugation in buffer to which increasing proportions of Freon were added produced viral preparations of the highest purity (9) (Fig. 1).

Preparations for electron microscopy were made either directly by placing of a droplet of virus suspension on a Formvar and carbon-coated grid and letting the buffer salts dialyze out by floating the grid on distilled water (4), or, alternatively, they were prepared from extensively dialyzed suspensions (9, 10).

One series of preparations was air-dried from distilled water; another series was fixed with an ethyl alcohol-acetic mixture of 5 ml 95% ethanol, 1 ml H_2O, and 0.4 ml glacial acetic acid. The object carriers for electron microscopy were either 200 mesh copper grids or platinum or platinum-iridium one-hole disks.

Another series was treated with 0.02% pepsin in HCl at pH 2.0 or at pH 0.5 for 20 h at 37°C or with HCl at the two pH levels alone. An additional series was treated with 0.02% trypsin in 0.15 M phosphate buffer, pH 7.0, for 48 h at 37°C. And finally uranyl acetate was used at two concentrations as a positive stain.

Electron Microscopy

Areas of evenly dispersed virus particles were electrographed in an RCA-4B electron microscope modified to provide a differential vacuum close to 10^{-8} Torr at the specimen level and around the objective contrast aperture. An orb-ion pump, connected to special chambers that replaced regular microscope components, provided the high vacuum. With this device there is a contamination rate of <0.06 Å/s.[2] Recently, we have also the Zeiss EM 10, fitted with two ion-getter pumps, and a contamination rate of 0.001 Å/s when high vacuum of 10^{-7} Torr plus a cold trap was used as an anticontamination device.[1] A sensor, 1 cm^2 in size and mounted at the level of the flourescent screen, was coupled to a microammeter. In the two instruments, the electron beam intensity in a particle-free area of

[2]Bahr, G. F., A. Collins, W. F. Engler, and J. Reisner. 1978. Experiences with an electron microscope operating at 10^{-7} to 10^{-8} Torr at specimen and contrast aperture level. Unpublished data.

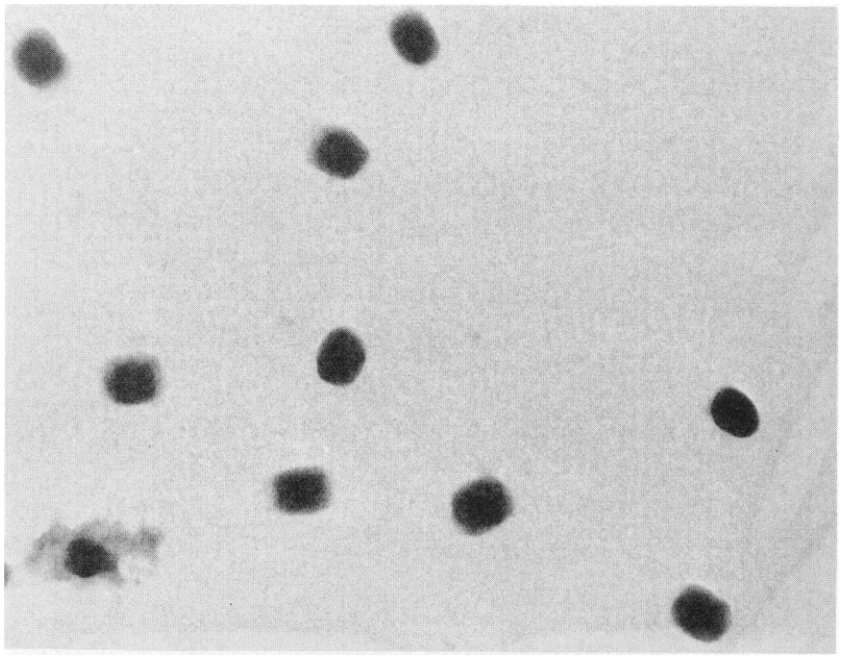

FIGURE 1 Representative area of an air-dried vaccinia virus preparation used in this study for the determination of the dry mass of individual virions by electron microscopy. ×60,000. (AFIP Neg. 78-6597-1.)

the preparation was set to a constant background value of 3.1×10^{-13} amps by spreading the beam with an overfocused condensor system, which remains in our experience remarkably constant over 5–10 min. The selected field of the preparation was recentered if necessary; a photographic plate was exposed for precisely 2 s. Magnifications close to ×15,000 were employed throughout.

Underfocus up to 0.25 μm renders clearly outlined objects and does not influence mass determinations to a significant degree (4). The total load of plates or film for the type of microscope used was exposed in rapid succession without lengthy refocusing and throughout at a rather low beam intensity. The photographic material was processed without delay under controlled conditions of time and temperature.

A technique for the determination of dry mass of objects from the contrast in electron micrographs has been developed by Bahr and Zeitler, and its theory and applicability have been studied in detail (4, 12, 13). The quintessence of the technique is that one can calculate the proportion of the dry mass of an object's point to its corresponding photometric transmission (over background) in the negative. The sum of the transmission of all image elements can be obtained by relatively simple optical integration, which renders a value that is directly proportional to the total dry mass of the object. Based on this principle, several generations of integrating photometers were built, the most advanced of which is the IPM-2 (Carl Zeiss, West Germany). For relative mass determinations, all that is required is the difference between two measurements of transmission, one of which is over the object and the other in the background (Fig. 2). For absolute values of mass, a set of calibration standards, usually polystyrene spheres, is electrographed, processed, and measured along with the objects. Also a precision magnification standard, such as the replica of an optical grating, is photographed with each set of object plates (4).

Large series of measurements can be carried out expeditiously on the IPM-2, especially if one takes advantage of the built-in electronic output features for recording data on magnetic or paper tape for computer processing. A total of 34,000 single measurements including repeats has been performed in connection with the present report.

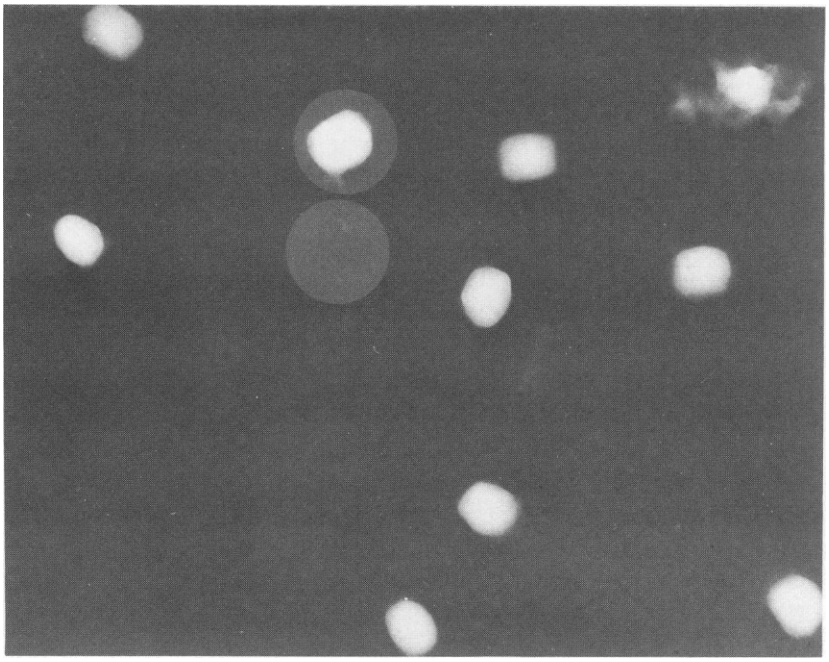

FIGURE 2 Montage demonstrating how transmission measurements over the image of a virion and over background are carried out on the negative of Fig. 1. In this way the difference between the transmissions, basic to the calculation of the dry mass of individual virions, can be obtained, while the operator is in the position of judging the quality of preparation (distribution and purity) as well as the preservation of the measured virion. ×60,000. (AFIP Neg. 78-6597-2.)

For about one-third of the series of electron micrographs, positive, enlarged prints were made for us to select and mark unambiguous images of virus particles as guidance during photometry. Also particles with extraneous material (Fig. 1) could thus be avoided. This rather time-consuming practice was abandoned when it was found that the decision could be made with equal certainty directly from the negative plate during photometry.

RESULTS

The determination of the distribution of mass in vaccinia populations was carried out on several principally separate preparations, of which five showed sufficiently pure virions and a suitable distribution on the carrier film. These are listed in Table I. Subfractions were analyzed by utilization of all grids available, which yielded the indicated number n of measured particles. A grand total of >7,800 virions constitutes the material on which the present results and interpretations are based.

The average median mass of 5.26×10^{-15} g is calculated as the weighted mean of determinations of subfractions. The standard deviation σ, also averaged over all subfractions, pertains to the logarithms of the weight values. The reason for this is that the distributions were found to obey a log-normal rather than a normal distribution. In fact, the weight, rather than the logarithms thereof, exhibits a somewhat skewed distribution with a predominance of heavier particles.

A typical example of a distribution of mass measurement on 854 air-dried virions is shown

TABLE I
DISTRIBUTION OF DRY MASS IN POPULATION OF UNFIXED VACCINIA VIRIONS, HAMBURG

Preparation	n	Median weight $\times 10^{-15}$ g First determination	Second determination	σ (Log scale) First determination
41	329	5.22		0.065
	342	4.95	4.97	0.070
	194	5.31		0.071
	801	5.07	5.23	0.062
	284	5.13		0.065
	854	5.23		0.070
45	249	5.15		0.068
	546	5.28		0.063
	283	5.93		0.055
	224	4.90	4.98	0.065
	239	5.29		0.050
46	193	5.13		0.065
	405	5.32		0.065
	309	5.36		0.063
	119	5.53		0.060
47	215	5.60		0.048
	355	5.53		0.051
	516	5.63	5.57	0.050
	106	5.31		0.060
	230	5.24		0.065
48	263	4.71		0.090
	226	5.14		0.085
Total	7282			0.064

Weighted mean, 5.26.

in the probability chart of Fig. 3. It can be seen that the data fit a straight line very well when the cumulative frequency of the logarithms of the masses of individual virions is plotted. The advantage of the probability chart is not only the ready confirmation of the normality (here log normality) of the distribution but also the direct wat the standard deviation σ can be read off as the difference between the 50, 16, and/or 84% values, that is, from the slope of the straight line. The lightest particles in this distribution were found to be close to 3.8×10^{-15} g, while the heaviest weighed 8×10^{-15} g; the former were <4% (34 virions) and the latter <0.5% (4 virions) present among the 854 particles measured.

All of the populations listed in Table I were comparable to the one depicted in Fig. 3, the slope of the plots being remarkably consistent as evidenced from the slightly varying σ.

For us to verify that the variation between mass of individual particles is intrinsic to the viral population and not artificially simulated, by erroneous measurements or other methodological factors, a set of standards, namely polystyrene spheres, were analyzed (13). Spheres were most suitable for this purpose because the diameters can be determined with great accuracy by a simple length measurement, the mass of the sphere being proportional to the volume. This of course presupposes knowledge of the magnification employed. Of each sphere, the photometric equivalent R (R = transmission T minus transmission T_0 of background) was compared with its mass value w as calculated from the diameter. The problem then was

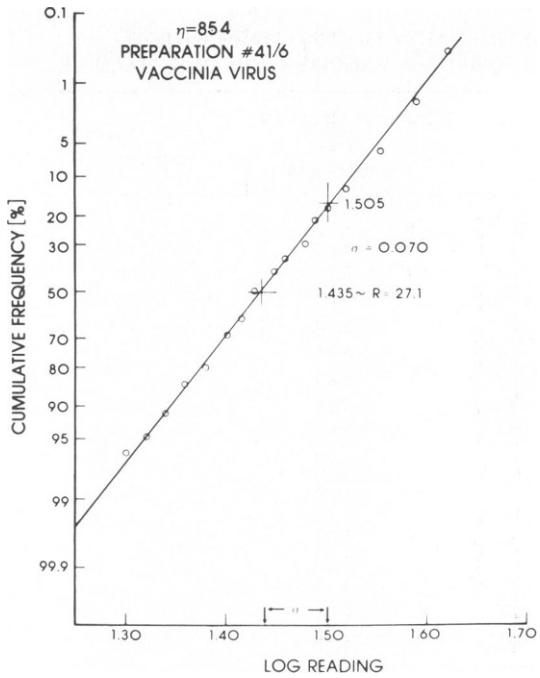

FIGURE 3 Weight values for a population of vaccinia virus are presented in a probability chart. In calculating the absolute mass W, R the photometer reading, is multiplied by a factor encompassing calibration and magnification (1). $R \times 0.193 \times 10^{-15}$ g = $W = 5.23 \times 10^{-15}$ g, $\sigma = 0.070$; for $R = 27.1$. Log reading = log base 10.

simply to determine the standard deviation of R for a given w and compare this standard deviation with the ones found for the mass distribution of the vaccinia virus.

The accuracy of the estimates was enhanced by assumption of a simple mathematical model for the distribution of the sphere mass. Instead of the ideal linear relationship, $R = Bw$ where B is a factor encompassing magnification, and other parameters making the photometric reading R comparable to the calculated mass of sphere w) the model contains an additional exponential factor $\exp(-Cw)$, which takes into account the following experimental facts. When the grid becomes too densely covered, so that several particles overlap, the dynamical range of the photographic plate is not sufficient to accommodate the range of transmission. The exponential factor then compensates for the overlap, and indeed for the extreme case of infinite mass, the photometric calibration would be zero.

The data had values of w ranging from nearly zero to 3,500 g $\times 10^{-12}$ (groups of spheres whose diameters could be individually determined and summed up), while the corresponding summed up R ranged from ~0 to 1,100.

Since the vaccinia virus particles, even in clusters of two or three, nowhere approached w values of 350, the calibration function $R = (A + Bw)\exp(-Cw)$ was estimated for a range of $w < 350$ by a modified, step-wise Gauss-Newton iteration. Also, it was deemed appropriate to correct the fit through the origin, so that when $w = 0$, $R = 0$. The form of the model with this restriction becomes $R = (Bw)\exp(-Cw)$.

Here are the estimates of the parameters and their asymptotic standard errors: parameter estimate, $B = 0.566$, $C = 0.0002677$; standard error, $B = 0.0120$, $C = 0.0001094$.

The standard error of estimate (or calibration), σ of R and σ of w was found to be 3.106 in the R scale or equivalently 0.025 in the logarithum scale.

It is appropriate to notice that the log equivalent standard error of calibration of 0.025 is roughly two-fifths of the value of 0.064 (Table I) of vaccinia virus. Should the range of w be restricted even further than 350 in the above calculations and be limited to 130, a value still in excess of that for vaccinia masses, then the standard error of calibration becomes 1.73 instead of 3.106, and its log equivalent standard error is 0.0142 instead of 0.025, viz., close to one-fifth of 0.064 of vaccinia virus.

This analysis suggests that populations of vaccinia virus possess distributions with greater variation of dry mass than can be accounted for by statistical errors of the measuring or the calibration procedure.

DISCUSSION

The range of $3.5–8 \times 10^{-15}$ g in which vaccinia varies is more than twofold. Deficiency in some protein or other component, except DNA, is compatible with function of species to a more limited extent than is surplus. For this reason it is intuitively plausible for the distribution to occur as or approach a log-normal distribution that allows for the occurrence of heavier individuals. This interpretation of the log-normal distribution is heuristic; the biologic origin of it, however, is likely to be complex metabolic equilibria within the host cell. Other pertinent studies on the variability of dry mass and of dimension are reported in publications that also review the applicable earlier literature (1, 2, 3).

Other possible mechanisms for the observed heterogeneity of vaccinia virions deserve attention. Nonvolatile salts of the buffer used may be uneavenly or nonrandomly absorbed by the virions. This appears, however, not to pose a serious limitation since there is a close correspondence of previously published results of mass determination by electron microscopy and different experimental procedures. One would also expect a significant increase of the heterogeneity for positively uranyl-stained particles if salt indeed were heterogeneously bound. This insignificant increase of σ for positively stained particles speaks against bound salts as being the reasons for mass heterogeneity. Particles might also lose parts of their outermost layers due to the mechanical forces acting during air drying or they might lyse during preparation and lose internal material. In the latter case a mass distribution skewed to lower values would result, in contradiction with the presented findings. The same would be the case if mass loss through electron bombardment had a differential effect on virions. Generally, overall mass loss is small for complex-biological structures (2).

A word about selecting virions for measurement and purity of the sample. Vaccinia virions were purified by repeated centrifugation in buffer, so that they could be studied by thin section electron microscopy and by various staining methods (9). The method of quantitative electron microscopy allows in addition the acceptance or rejection of individual virions by an experienced virologist (electron microscopist, D. Peters).

Short of resorting to biochemical methods of fractionation and chromatographic analysis,

TABLE II
VACCINIA VIRUS, HAMBURG. DRY MASS OF VIRIONS

	Preparation	n	Median weight $\times 10^{-15}$ g	σ (Log scale)
Acetic acid-ethanol. Mixed with 0.264-μm diameter polystyrene latex, for direct comparison.	41	728	3.46	0.090
		318	3.73	0.065
		748	3.62	0.073
		764	3.53	0.055
		353	3.74	0.090
	46	370	3.44	0.075
		368	3.08	0.060
	47	468	3.27	0.075
	48	214	3.89	0.108
		137	5.31	0.071
		105	5.48	0.051
Treated with hydrochloric acid	pH 2.0	705	5.59	0.047
		193	5.13	0.065
	pH 0.5	673	5.18	0.058
With pepsin treatment	pH 2.0	447	0.98	0.075
	pH 0.5	153	0.85	0.140
	pH 0.5	214	0.96	0.125
With trypsin treatment, pH 7.0, in 0.15 M phosphate buffer		148	0.760	0.128
		84	0.580	0.134
With uranyl acetate stain for 1 h	2%, pH 4.4	108	9.18	0.120
		177	9.20	0.129
		232	8.18	0.058
	10^{-4} M, pH 3.5	183	7.80	0.079

experiments were undertaken to assess possible qualitative differences underlying the quantitative heterogeneity of vaccinia virions.

Digestion with pepsin and trypsin at different pH, fixation in acetic acid-ethanol, or weak HCl treatment and positive staining with uranyl acetate were used as first approaches. Results are tabulated in Table II. Although the distribution of mass values broadened somewhat, one third of the σ remained comparable to the σ of untreated virions. This we interprete as indirect evidence that qualitative differences of hypothetical vaccinia subfractions do not give rise to the observed heterogeneity.

A look at other strains of vaccinia and viruses of comparable size and mass, e.g., myxoviruses suggested (Table III) that distributions similar to the vaccinia investigated, i.e., a log normal distribution is applicable to these strains. The mass values of untreated virions from Hamburg (Table II) are very similar to previously published values. Jokklik (7) found 5.5×10^{-16} g, with 5% of the dry mass being DNA—2.75×10^{-16} g or 2.44×10^{-16} g, according to Becker and Saro (5). Marquardt et al. (9) reported 5.69×10^{-15} g dry mass and a volume of 8.37×10^{-15} cm^3. Both laboratories determined the mean of the population,

TABLE III
MEDIAN OF DRY MASS OF OTHER POX VIRUSES

Preparation	n	Median weight $\times 10^{-15}$ g	σ (log scale)
Vaccinia, Lea (7 *N*)	158	6.17	0.114
Vaccinia, W. R.	109	5.98	0.095
Vaccinia, W. R.	150	6.04	0.090
Cowpox	156	6.50	0.113
Rabbitpox	79	5.31	0.124
Fowlpox, Australia	176	6.02	0.090
Fowlpox, Mississippi	164	7.22	0.120
Myxoma	135	6.22	0.112

which is for a skewed distribution slightly higher than the median mass reported in this paper.

An experiment was also undertaken to determine the mass of vaccinia virions in the presence of polystyrene spheres, i.e., both had been exposed to the same drying conditions (Table II). The result for virion mass is quite comparable to those listed in Table I. Such preparations cannot be used extensively since latex and virions tend to form aggregates. Therefore, all other measurements were performed on virions and latex-spheres prepared comparably but separately.

This work was completed while G. F. Bahr was the recipient of an award from the Alexander von Humboldt Foundation, Bad Godesberg, Germany. The shipment of a preparation of myxoma virus from the laboratory of Dr. W. R. Sobey of the Division of Animal Genetics, C.S.I.R.O., Sidney, Australia, and the one of fowlpox virus from the laboratory of Dr. C. C. Randall, University of Mississippi Medical Center, Jackson, Mississippi, are gratefully acknowledged. We are indebted to Dr. W. K. Jokklik for discussions in the early phases of this project. The electrography and the measurement of the large numbers of virions would not have been possible without the skillfull assistance of U. Mikel, W. F. Engler, and A. Collins.

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